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Evaluation of α-Amylase Assays with 4-Nitrophenyl-α-oligosaccharides as Substrates

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Summary: Measurements of α-amylase with 4-nitrophenyl glucosides offer the following advantages over methods that rely on the formation of NADH:

a short lag phase, no apparent interference by metabolites and enzymes of the sample and extremely stable substrates with low blank values. The intrinsic sensitivity of nitrophenol formation was equal to that of hydrolysis of maltotetraose, but was less than that of glucose-producing methods using oligosaccharides. In contrast to starch, the chromogenic substrates are more rapidly hydrolysed by salivary than by pancreatic amylase.

Disadvantages of these substrates are:

higher turnover by animal than by human amylases, and a marked susceptibility of the chromophore to small changes of pH and protein concentration. Some analytical qualities such as specificity, accuracy, precision, stability of the substrate and linear range are described in detail and compared with those of other methods.

Bewertung von Methoden zur Bestimmung von α-Amylase mit 4-Nitrophenyl-α-oligosacchariden als Substraten

Zusammenfassung: α-Amylase-Bestimmungen mit 4-Nitrophenylglucosiden sind Methoden, die auf einer Reduktion von NAD beruhen, durch eine kürzere lag phase, fehlende Störung durch Metaboliten und Enzyme der Probe und durch außerordentlich haltbare Substrate mit niedriger Leerreaktion überlegen. Die Empfindlichkeit der Nitrophenolbildung entspricht der Spaltung von Maltotetraose, sie bleibt jedoch hinter dem Entstehen von Glucose aus Oligosacchariden zurück. Im Gegensatz zu Stärke werden die chromogenen Substrate schneller durch Speichel- als durch Pankreasamylase abgebaut.

Nachteile ihrer Verwendung sind ein höherer Umsatz durch Amylasen tierischer Herkunft im Vergleich zu menschlichen Enzymen und der starke Einfluß geringer Veränderungen der Proteinkonzentration und des pH-Wertes im Ansatz. Im einzelnen werden die Spezifität, Richtigkeit und Präzision dieser Verfahren, die Haltbarkeit ihrer Substrate, der lineare Meßbereich und ein Vergleich mit anderen Methoden beschrieben.

Introduction

The growing number of continuous measurement techniques for the determination of α-amylase (1,4-α-*D*-glucan glucanohydrolase, EC 3.2.1.1) activity has been increased by the development of chromogenic methods based on the release of 4-nitrophenol. The original substrate α-(4-nitrophenyl)-maltoside was highly insensitive toward the activity of sera (1, 2), and the preparation of 4-nitrophenyl-α-oligosaccharides (3, 4) remained difficult till the enzymic synthesis described by *Wallenfels* et al. (5, 6). But the use of these substrates has now become increasingly popular; and, as denoted by a series of abstracts (7–17), it has resulted in the production of test kits, whose components cannot be obtained separately. Hence a systematic evaluation of the ana-

lytical variables is impossible. The following investigation is a test of the reliability of two chromogenic α-amylase assays in comparison with other commercially available test kits.

Materials and Methods

Apparatus and reagents

Absorbance measurements were made with a Hg-line photometer 1101 M Eppendorf, and the reaction rates were monitored at 334, 365 and 405 nm with a potentiometric recorder 4412 (both from Netheler & Hinz, Hamburg, Germany). While the spreading of the absorbance (b = 200 mm representing $A_0 - A_{1.0}$) was kept constant, the velocity of the recorder chart (v = x mm/min) was adjusted so that the slope of the reaction rate curve versus time remained between 14 and 40° to minimize errors in measuring the angle and its resulting tan α. All data originated from duplicates that differed not more than 1°. The catalytic concentration was calculated according to

$$U/l = \tan\alpha \cdot \frac{v}{b} \cdot \frac{10^6}{\varepsilon \cdot 1} \cdot \frac{FV}{SV} \cdot f$$

from tan α, v, b, molar absorption coefficient (ε in $l \cdot mol^{-1} \cdot mm^{-1}$), path length of the cuvette (d = 10 mm), f (a dilution factor for samples with high activities), final (FV) and sample volume (SV).

We investigated clear, lipaemic and icteric sera immediately after blood clotting and urine specimens within four hours after micturition. Following tests were applied:

(A) Hydrolysis of 4-nitrophenyl oligosaccharides involving the action of α-glucosidase:

(1) BM α-Amylase Enzymatic Colorimetric Test® (Boehringer, Mannheim, Germany; (17)) with 4-nitrophenyl-α-*D*-maltoheptaoside and

(2) Testomar®-Amylase (Behringwerke AG, Marburg, Germany) or Pantrak™ E. K. Amylase (Calbiochem-Behring Corp., La Jolla, CA 92037; (11–15)) with 4-nitrophenyl-α-*D*-maltopentaoside plus 4-nitrophenyl-α-*D*-maltohexaoside.

(B) Cleavage of maltotetraose with maltose phosphorylase, β-phosphoglucomutase and glucose-6-phosphate dehydrogenase as auxiliary enzymes in Monamyl-neu® (Biomed, München, Germany) or Amylase-DS® (Beckman Instruments Inc., Carlsbad, CA 92008; (18)).

Some properties of α-amylase were comparatively investigated by an amyloclastic procedure (19), and most of the analytical qualities of the aforementioned methods were tested with two further kits based on the formation of glucose by

(C) Degradation of oligosaccharides with subsequent turnover by α-glucosidase, hexokinase and glucose-6-phosphate dehydrogenase:

(1) BM α-Amylase UV-Test® (Boehringer; (20)) with maltoheptaose and

(2) M+D–UV-Amylase® (American Hospital Supply, München, Germany; (21) modified according to l.c. (22)) with oligosaccharides (and removal of endogenous glucose).

Human salivary and pancreatic amylase were isolated as previously described (23). We used α-glucosidases (α-*D*-glucoside glucohydrolase, EC 3.2.1.20) from human renal cortex as a particle free homogenate supernatant (100000 *g* × 60 min) and from yeast (Boehringer, Mannheim). Both proved free from α-amylase activity and inhibitors when tested by an amyloclastic method (19). 4-Nitrophenol was twice recrystallized from water. Enzymes were purchased from Boehringer (Mannheim) and all other chemicals from Merck (Darmstadt, Germany) in best available grade, except Acarbose[1]) and the infusion solutions of various producers.

[1]) We are indebted to Dr. *W. Puls* (Bayer AG) for the generous gift of Acarbose (BAY g5421).

Procedures

We prepared all reagents with doubly distilled water. The molar absorption coefficients of 4-nitrophenol were determined from 500 μmol/l solutions in buffers according to the specification of the manufacturers. Temperature and pH-value were controlled within the cuvette in assays without and with addition of human serum albumin corresponding to the volume fraction of the sample.

To check the influence of drugs and metabolites on the enzyme assay we added these substances instead of (positive interference) and in addition to the sample (negative interference). All determinations were carried out manually with sample volumes of 20 μl (A1, A2, B) or 10 μl (C1, C2) as micromethods acording to the kit inserts.

To compare results obtained with the individual methods, we used nonparametric techniques (*Wilcoxon*'s signed ranks test), t-test for paired samples and parametric tests after log transformation of the data to achieve a *Gaussian* distribution.

Results and Discussion

Influence of the reaction mixture on activity calculations

The molar absorptivity of 4-nitrophenol between pH 6.5 and 8.0 was highly dependent on small variations of hydrogen ion concentration, temperature and sample constituents. The values for $\varepsilon_{405\,nm}$ differed with respect to the protein content of the reaction mixture; e.g. the presence of albumin slightly increased the absorbance in N-2-hydroxyethylpiperazine-N′-ethanesulphonic acid, whereas the absorbance in phosphate was markedly diminished (tab. 1). Those effects were not negligible as stated by others (26). This has to be considered when comparing results from sera, diluted sera and other biological fluids. Probably, the biosynthesis of ω-nitrostyryl glucosides might minimize this problem because of the high absorption of the liberated chromophore above pH 5.0 (27).

Reaction rates

The reaction lag phases decreased with increasing temperature and apparently varied with the matrix of the sample (tab. 2). Nevertheless, in contrast to amyloclastic techniques (19), there always remained a short time of delayed catalysis, which can be most probably ascribed to the properties of the auxiliary enzymes or to their inhibition by low molecular weight constituents. In particular, the extended lag phases in urines with bacterial contamination were reduced by dialysis of the specimens against potassium phosphate, 5 mmol/l, pH 7.5. Dilution of the specimen was less effective.

Even after the lag phase the catalytic concentrations of sera, sterile urines and control material varied slightly, depending on the reaction time. These differences originating from various determinations be-

Tab. 1. Molar absorption coefficient of 4-nitrophenol (mean and standard deviation from 10 determinations) in comparison with reported values. Human serum albumin, 1.92 g/l, corresponds to 50 g/l within the sample. For further details see methods.

Conditions	Temperature	$\varepsilon_{405\,nm}$ ($l \cdot mol^{-1} \cdot mm^{-1}$) $\bar{x} \pm SD$	reported	Reference
Potassium phosphate, 50 mmol/l sodium chloride, 50 mmol/l pH 7.1	25 °C 30 °C	981 ± 6 1025 ± 6		
Potassium phosphate, 50 mmol/l sodium chloride, 50 mmol/l human serum albumin, 1.92 g/l pH 7.1	25 °C 30 °C	941 ± 5 952 ± 5	900 950	(17)
N-2-Hydroxyethylpiperazine-N'-ethanesulphonic acid, 48 mmol/l pH 7.0	25 °C 30 °C	804 ± 4 808 ± 4	800 800	(24)
N-2-Hydroxyethylpiperazine-N'-ethanesulphonic acid, 48 mmol/l sodium chloride, 48 mmol/l human serum albumin, 1.92 g/l pH 7.0	25 °C 30 °C	810 ± 5 828 ± 8	870	(15)
Sodium hydroxide, 10 mmol/l	25 °C	1835 ± 10	1838	(25)
Sodium hydroxide, 100 mmol/l	30 °C	1845 ± 10	1848	(24)

Tab. 2. Reaction lag phases of α-amylase from different origins at 25 °C and 30 °C.

	4-nitrophenyl-α-*D*-malto-heptaoside (A 1)	4-nitrophenyl-α-*D*-malto-pentaoside/ hexaoside (A 2)	Malto-tetraose (B)
Pancreatic amylase			
25 °C	3 min	3 min	4 min
30 °C	2.5 min	2.5 min	2 min
Salivary amylase			
25 °C	4 min	5 min	7 min
30 °C	3 min	2.5 min	3 min
Normal sera (n = 10)			
25 °C	7 ± 1 min	7 ± 1 min	10 ± 2 min
30 °C	5 ± 1 min	5 ± 1 min	8 ± 1 min
Macroamylase sera (n = 5)			
25 °C	4 ± 1 min	5 ± 1 min	8 ± 2 min
30 °C	3 ± 1 min	3 ± 1 min	6 ± 1 min
Urines (n = 10)			
25 °C	7 ± 1 min	7 ± 1 min	11 ± 3 min
30 °C	5 ± 1 min	5 ± 1 min	8 ± 1 min
Precinorm U® (porcine pancreatic amylase)			
25 °C	7 min	6 min	10 min
30 °C	5 min	4 min	6 min

tween 5 and 20 min of reaction were rather small on average, but were sometimes significant (tab. 3). Therefore, all further calculations were based on measurements between 15 and 20 min of incubation with maltoheptaose and oligosaccharides and between 10 and 12 min with the other substrates. The concentration of α-amylase obviously did not cause these variations.

Interferences

Previous studies (4, 28–30) demonstrated that measurements of α-amylase via NADH formation were affected by α-glucosidase and by some metabolites, especially in urines. However, the quality of those test kits has improved, and only a few batches of test B (maltotetraose) were susceptible to pyruvate, 2

Tab. 3. Relative catalytic concentration of α-amylase in sera at 25 °C. The data were derived from measurements of 25 samples at different intervals from the start of the reaction (10 min = 1.000): mean ± standard deviation, probability of error (two sided tested) of differences between values at 10 min and other reaction times.

Substrate (test)	Time of measurement after addition of the sample 5 min	Range at 10 min	15 min	20 min
4-Nitrophenyl-α-*D*-malto-heptaoside (A 1)	0.991 ± 0.001 p < 0.001	28.3 – 1410 U/l dilution above 500 U/l	1.013 ± 0.002 p < 0.20	1.001 ± 0.004 p < 0.40
4-Nitrophenyl-α-*D*-malto-pentaoside and -hexaoside (A 2)	0.983 ± 0.002 p < 0.05	13.3–1600 U/l dilution above 500 U/l	1.022 ± 0.003 p < 0.10	1.012 ± 0.003 p < 0.40
Maltotetraose (B)	0.988 ± 0.003 p < 0.02	11.4–408 U/l dilution above 250 U/l	1.008 ± 0.003 p < 0.40	1.010 ± 0.002 p < 0.40
Maltoheptaose (C 1)	not determined	25.0–1580 U/l dilution above 500 U/l	1.032 ± 0.003 p < 0.005	1.040 ± 0.003 p < 0.005

mmol/l, which decreased normal amylase values by nearly 10%. We did not observe any interference by fructose or acetoacetate in all methods presented in table. 4. The slight decrease of 4-nitrophenyl-α-*D*-maltopentaose/-hexaose hydrolysis by glucose, 55 mmol/l, paralleled that of 4-nitrophenyl-α-*D*-maltotetraose (16) and could be overcome by an increased substrate supply. The inhibitory effect of hydroxyethyl starch must be considered in view of the long known adsorption of α-amylase by starch (31).

Since the lag phase of the reaction sequence releasing 4-nitrophenol largely depends on the concentration of α-glucosidase within the assay system (32, 33, 34), we tried to abridge this phase by addition of α-glucosidase from yeast. However, neither the lag phase nor the net absorbance increase in the linear portion of the activity curve were influenced by this enzyme up to 250 kU/l within the assay mixture, thus confirming the observations of *David* (32) and *Rauscher* et al. (17) who used 4-nitrophenyl glucosides for the measurement of α-amylase activity. The same was true for the hydrolysis of maltoheptaose in test C1 and that of oligosaccharides in test C2, whereas the apparent α-amylase concentration was overestimated by 10% in the maltotetraose procedure, probably by the increased production of maltose from maltotetraose (30).

α-glucosidase from human kidney did not influence the tests A-C, even in concentrations up to 60 U/l within the sample, thus exceeding the normal range of 2–23 U/l (35) in human urine. Only extreme concentrations, which might be encountered in acute renal necrosis, interfered in the release of 4-nitrophenol: 677 U/l of α-glucosidase, determined according to *Bonini* et al. (36), were determined as 76.3 U/l (test A1) or 35.1 U/l (test A2) of α-amylase.

From experiments with porcine enzymes (37), acarbose is known to be a more potent inhibitor of maltase and sucrase then of α-amylase. *Suehiro* et al. (38) reported a noncompetitive inhibition of human amylases by acarbose, a complex oligosaccharide, which might be possibly resorbed in minimal concentrations from the gut. Therefore, after an incubation with acarbose, 20 mg/l, at 25 °C for 20 min, the residual activities of purified human amylases (measured with starch in an amyloclastic method (19)) and of α-glucosidases from yeast (determined with 4-nitrophenyl-α-*D*-glucopyranoside, 2 mmol/l, in N-2-hydroxyethylpiperazine-N′-ethanesulphonic acid, 50 mmol/l pH 7.0 and potassium phosphate, 50 mmol/l, pH 7.1, corresponding to the buffers of tests A1 and A2) were assayed: 0.94 of α-glucosidase, but only 0.43 of pancreatic and 0.66 of salivary α-amylase activity were retained. In the presence of acarbose, 240 mg/l, the residual activities amounted to 0.93, 0.03 and 0.09, resp., i.e. acarbose preferentially acted on α-amylase, while its influence on the auxiliary enzyme in glucosidase-mediated assays was minimal.

Specificity

The 4-nitrophenyl glucosides used in tests A1 and A2 were neither hydrolysed by sera whose amylase activity was abolished by ethylenediaminetetraacetic acid, 2 mmol/l, nor by 80 U/l of α-mannosidase, α-

Tab. 4. Influence of diverse metabolites and infusion media on four α-amylase assays.
The first figure indicates the effect of the solution tested (20 µl instead of serum) on the assay system (control: water = 0). The second number denotes the influence of the interfering substance on the determination of α-amylase at 25 °C (control: serum = 1.0).

	4-Nitrophenyl-α-*D*-maltoheptaoside (A 1)		4-Nitrophenyl-α-*D*-maltopentaoside/ hexaoside (A 2)		Maltotetraose (B)		Maltoheptaose (C 1)	
Serum	288 U/l		146 U/l		86.3 U/l		295 U/l	
Pyruvate, 2 mmol/l	0	0.99	0	1.01	0	0.9–1.0	0	1.01
Lactate, 10 mmol/l	0	1.02	0	0.99	0	0.98	0	0.97
Maltose, 5 mmol/l	0	1.02	0	0.95	0	1.0	0.02	0.97
Glucose, 10 mmol/l	0	1.01	0	1.0	0	1.0	0	1.0
55 mmol/l	0	1.01	0	0.95	0	0.98	0	0.94
Thomadex 40®, 80 g/l	0	0.99	0	0.97	0	0.96	0	1.0
Haemaccel®, 7 g/l	0	1.02	0	0.98	0	0.96	0	0.99
Hydroxyethyl starch, 20 g/l	0	0.83	0	0.79	0	0.68	0	0.90
Mannitol, 40 g/l	0	0.93	0	0.97	0	0.93	0	0.97
Aminomel LX6®, 16 g/l[1])	0	1.0	0	0.98	0	0.90	0	1.01

[1]) contains malate 34 mmol/l, *L*-amino acids, KCl, $Mg(CH_3COO)_2$ and NaOH

galactosidase, β-galactosidase and β-glucosidase, added to the reaction mixture.

Table 5 summarizes the relative activities of human α-amylases measured by different methods. These results support findings of *Meier* et al. (29) and of *Sampson* et al. (39), who observed that maltotetraose was attacked equally by salivary and pancreatic amylase, whereas starch favoured the action of the pancreatic isozyme. The nitrophenyl oligosaccharides held an intermediate position with relative low activation energies for salivary amylase. From a diagnostic point of view a substrate with a long carbohydrate chain would be desirable to minimize the attack by extrapancreatic amylases.

Tab. 5. Intermethod comparison of relative reaction rates of crystallized human α-amylases (23).
The relative activity of the pancreatic isozyme at 25 °C was fixed to 1.00 in all assays, the relative activity of salivary amylase to 1.00 in test B with maltotetraose. The ciphers mark conversion factors for 30 °C and 37 °C, activity ratios (pancreatic amylase/salivary amylase), activation energies (according to the empirical *Arrhenius* equation), and Q_{10} values (calculated from measurements between 25 °C and 37 °C).

	25 °C	30 °C	37 °C	Activation energy (kJ/mol)	Q_{10}
4-Nitrophenyl-α-*D*-maltoheptaoside (A 1)					
Pancreas	1.00	1.29	1.53	27.3	1.43[1])
Saliva	0.68	0.76	0.88	17.0	1.25[1])
Ratio P/S	1.47	1.70	1.74		
4-Nitrophenyl-α-*D*-maltopentaoside/-hexaoside (A 2)					
Pancreas	1.00	1.27	1.58	29.2	1.46[1])
Saliva	0.68	0.74	0.94	20.6	1.31
Ratio P/S	1.47	1.72	1.68		
Maltotetraose (B)					
Pancreas	1.00	1.32	1.80	37.7	1.63
Saliva	1.00	1.18	1.57	28.7	1.45
Ratio P/S	1.00	1.12	1.15		
Starch (19)					
Pancreas	1.00	1.18	1.45	23.7	1.36
Saliva	0.40	0.52	0.72	37.2	1.62
Ratio P/S	2.50	2.29	2.03		

[1]) Linearity between 30 °C and 37 °C uncertain

The same problem occurred with control sera as already stated for some other substrates (29, 40). Table 6 lists the differences of relative reaction velocities among control materials from human, porcine, and bovine sources, viz. the rapid breakdown of nitrophenyl glucosides by sera of animal origin and the slow breakdown by human specimens. Implications for the quality control of α-amylase assays have been previously reviewed by *Bretaudiere* et al. (41).

Analytical range, accuracy and precision

The manufacturers' claims for linear response in microassays with sera could be verified on the whole. We observed zero-order kinetics up to 1000 U/l (7.7 times the upper reference range) for test A1 with 4-nitrophenyl-α-*D*-maltoheptaoside at 25 °C. The corresponding limits were 370 U/l (7.4 times) for test A2 using 4-nitrophenyl-α-*D*-maltopentaoside/-hexaoside, 300 U/l (8.6 times) for test B with maltotetraose, 890 U/l (7.1 times) for test C1 with maltoheptaose, and 1000 U/l (10 times) for test C2 with oligosaccharides. On the other hand, the sample vo-

Tab. 6. Intermethod comparison of α-amylase assays with quality-control specimens at 25 °C.
The relative activities are presented in reference to results obtained with the maltotetraose method (test B = 1.0). The ciphers in brackets indicate their relative deviation from the assigned values (= 1.00, no assigned value ≙ −).

	4-Nitrophenyl-α-*D*-maltoheptaoside (A 1)		4-Nitrophenyl-α-*D*-maltopentaoside/hexaoside (A 2)		Maltotetraose (B)		Maltoheptaose (C 1)	
Control serum and batch no.								
Precinorm U® 1-572	10.1	(1.08)	5.23	(−)	1.0	(−)	9.35	(1.00)
Precinorm E® 1-366	16.7	(1.08)	8.80	(−)	1.0	(−)	15.5	(1.00)
Precipath E® 5-338	17.3	(1.07)	8.94	(−)	1.0	(−)	16.2	(1.00)
Monitrol I-E® 164[1])	3.73	(1.06)	1.65	(−)	1.0	(1.04)	3.43	(0.94)
Monitrol II-E® 64[1])	4.22	(1.05)	1.91	(−)	1.0	(1.04)	4.11	(1.03)
Validate N® 2018090[1])	3.78	(1.04)	1.77	(−)	1.0	(1.14)	3.96	(1.09)
Hyland P® P 21 B	10.5	(1.07)	5.44	(−)	1.0	(−)	9.97	(1.01)
Versatol E® 4 H 726	10.4	(1.05)	5.59	(−)	1.0	(1.10)	9.65	(0.97)
Kontrollogen LP® 623205 A[1])	3.60	(1.05)	1.63	(−)	1.0	(1.11)	3.46	(1.00)
Kontrollogen L® 623116[1])	3.63	(1.15)	1.55	(−)	1.0	(1.05)	3.49	(1.11)
Enza-Trol® ET 247 A	16.6	(1.18)	7.51	(−)	1.0	(1.00)	14.4	(1.06)
Seronorm® 147	12.6	(1.09)	6.81	(−)	1.0	(−)	12.2	(1.06)

[1]) Material of apparently human origin

lume fraction noticeably influenced the measured catalytic concentration only in tests with nitrophenyl glucosides. A ratio of sample volume to final volume of 1/51 yielded 8 ± 3% (n = 25) higher values than a 1/26 ratio. This agreed well with observations in highly active sera after dilution with sodium chloride, 155 mmol/l. By dilution of serum with 1 part of saline the relative activity increased from 1.0 to 1.12 ± 0.04 (A1: 4-nitrophenyl-α-*D*-maltoheptaoside, n = 8) and 1.04 ± 0.02 (A2: 4-nitrophenyl-α-*D*-maltopentaoside/-hexaoside, n = 8), by dilution with 9 parts of saline the respective data were 1.18 ± 0.07 and 1.06 ± 0.05. A dilution with potassium phosphate, 50 mmol/l, pH 7.1 (for test A1) or N-2-hydroxyethylpiperazine-N′-ethanesulphonic acid, 50 mmol/l, pH 7.0 (for test A2) produced identical values, which proved significantly different ($p < 0.05$) from the original relative activities. This was at variance with results obtained when human serum albumin, 50 g/l, was used as a diluent. With both tests A1 and A2 only a small increase was observed: to 1.02 ± 0.04 with a 1 plus 1 and to 1.05 ± 0.05 with a 1 plus 9 dilution.

Therefore, the stronger dilution effects of saline and buffer in test A1 were attributed to the higher absorption coefficient of 4-nitrophenol in phosphate buffer with decreasing protein concentrations as reported above. The opposite effect in N-2-hydroxyethylpiperazine-N′-ethanesulphonic acid evidently compensated to some extent the dilution of α-glucosidase inhibitors in both tests. This was concluded from a small rise of activity to 1.03 ± 0.04 by dilution with 9 parts of saline, phosphate buffer or human serum albumin in test C1 with oligosaccharides, while both the maltotetraose and the amyloclastic methods were not affected.

Tab. 7. Imprecision of α-amylase assays with 4-nitrophenyl-α-*D*-glucosides in manual performance at 25 °C. For further details, see methods.

Human serum pool	4-Nitrophenyl-α-*D*-maltoheptaoside (A1)		4-Nitrophenyl-α-*D*-maltopentaoside/hexaoside (A2)	
	Mean, U/l	CV, %	Mean, U/l	CV, %
	Imprecision within-run (n = 30)			
Normal range	65.2	3.62	27.9	3.38
Upper reference range	139	3.63	59.3	2.95
Pathological range	491	2.54	216	2.86
	Imprecision day-to-day (n = 20)			
Normal range	66,4	3.87	28.5	4.04
Upper reference range	145	3.19	60.6	2.79
Pathological range	494	2.94	218	3.06

The accuracy of four tests was checked with control sera whose assigned values were taken as 1.00. The corresponding data given in table 6 showed a slight positive bias of about 6% on average. This might originate from an inaccurate sample volume in the micromethod used. Table 7 summarizes the results of precision studies with chromogenic tests A1 and A2. Their imprecision was equal to that of the maltotetraose procedure (30), but less than that of assays with maltoheptaose and oligosaccharides. Their coefficients of variation (human serum pool in the pathological range, within-run, n = 30) were 3.22% for test C1 and 6.32% for test C2, probably due to their smaller sample volumes. In general the results agreed well with those reported recently (4, 17, 42, 43).

Stability of reagents and intrinsic sensitivity

The cleavage by α-glucosidase of glucosides and 4-nitrophenyl glucosides increases with decreasing chain length of the substrate (32, 33). Since α-glucosidase degrades the original substrates to smaller ones which are then hydrolysed more rapidly, the blank reaction accelerates with time after mixing substrate with auxiliary enzyme (tab. 8). Test A1 is also proffered with 4-nitrophenyl maltoheptaoside and α-glucosidase in separate vials. Nevertheless, even solutions ready for use containing nitrophenyl glucosides may be stored for 48 h at 4 °C or for 8 h at 25 °C if proper corrections are made for the blank reaction. We obtained identical results in assays with reagents just reconstituted, and with solutions kept

Tab. 8. Blank reaction rates of three α-glucosidase-mediated α-amylase assays.
The change of absorbance per hour was recorded during different periods after preparing the reagent containing substrate, auxiliary enzyme, buffer, and sodium chloride.

Period	4-Nitrophenyl-α-*D*-maltoheptaoside (A1)		4-Nitrophenyl-α-*D*-maltopentaoside/hexaoside (A2)		Maltoheptaose (C1)	
4 °C	ΔA_{405}/h	U/l	ΔA_{405}/h	U/l	ΔA_{366}/h	U/l
3– 4 h	0.002	0.289	0.001	0.054	0.020	3.30
7– 8 h	0.008	1.16	0.002	0.108	0.030	4.95
23–24 h	0.012	1.73	0.008	0.433	0.048	7.92
47–48 h	0.031	4.48	0,020	1.08	0.071	11.7
25 °C						
0– 1 h	0.009	1.30	0.009	0.488	0.062	10.2
3– 4 h	0.018	2.60	0.023	1.25	0.096	15.8
7– 8 h	0.031	4.48	0.041	2.22	0.124	20.5
11–12 h	0.042	6.07	0.052	2.82	0.156	25.7
15–16 h	0.050	7.22	0.061	3.30	0.194	32.0

as described above for α-amylase concentrations up to 500 U/l with test A1 and up to 200 U/l with test A2. On the other hand, the blank reaction of test C2 (not shown in table 8) was 16.6 ± 2.4 U/l (n = 10) immediately after preparing the reagent.

Concerning the quantitative signal, calculated in $\Delta A \cdot 1 \cdot \mu mol^{-1}$ (i.e. ΔA/min per U/l for an identical final to sample volume ratio), there was no essential difference in sensitivity between the cleavage of maltotetraose and the hydrolysis of chromogenic substrates. Taking test B with maltotetraose (334 nm) as 1.00, the relative intrinsic sensitivities were 1.00 ± 0.03 for 4-nitrophenyl-α-*D*-maltoheptaoside (405 nm), 1.09 ± 0.05 for 4-nitrophenyl-α-*D*-maltopentaoside/-hexaoside (405 nm), 6.69 ± 0.10 for maltoheptaose (334 nm) and 3.57 ± 0.26 for oligosaccharides (334 nm) derived from the determination of 10 sera ($\bar{x}$ ± s) over a wide range of α-amylase concentrations.

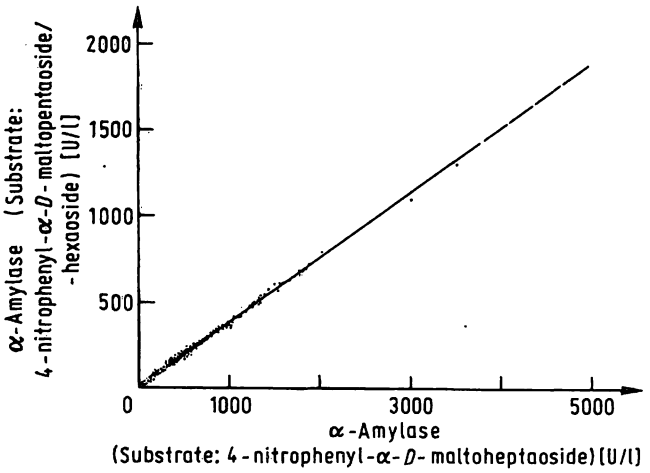

Fig. 1. Comparison of α-amylase determination by 4-nitrophenyl-α-*D*-maltoheptaoside (A1, abscissa) and 4-nitrophenyl-α-*D*-maltopentaoside/-hexaoside (A2, ordinate) with 120 sera at 25 °C: r = 0.996, y = 0.379x + 5.69 (standardized main component: y = 0.377x + 5.35).

Intermethod comparison

A practical evaluation was carried out with a total of 180 sera including lipaemic, icteric and haemolytic specimens and with 50 urines of healthy and diabetic persons. Both chromogenic methods were insensitive to lipaemia even with chylous samples containing 50 mmol/l of triglycerides; they were also insensitive to bilirubin up to 280 μmol/l in icteric sera and to haemoglobin concentrations between 0.5 and 2.7 g/l. As in the test with maltotetraose, both chromogenic methods were only limited by the capability of the photometer to measure high absorptivities. In urines glucose up to 330 mmol/l, after appropriate dilution to 170 mmol/l for test A1 and 112 mmol/l for test B, did not influence these assays, whereas α-amylase concentrations in test A2 were underestimated by about 7%, hence requiring a further dilution.

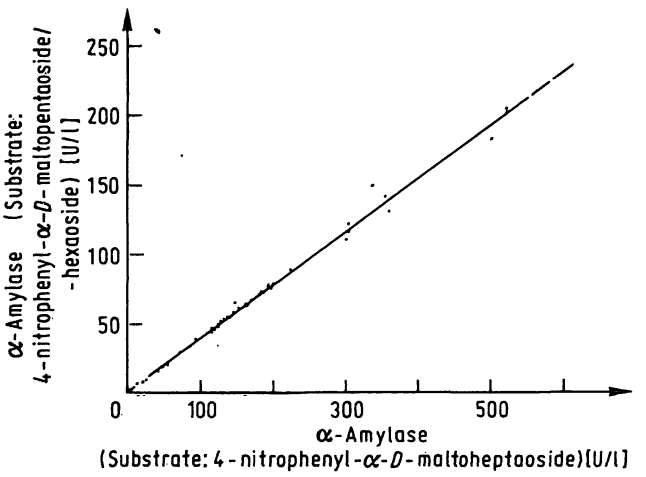

Fig. 2. Comparison of α-amylase determination by 4-nitrophenyl-α-*D*-maltoheptaoside (A1, abscissa) and 4-nitrophenyl-α-*D*-maltopentaoside/-hexaoside (A2, ordinate) with 45 urines at 25 °C: r = 0.970, y = 0.383x + 1.79 (standardized main component: y = 0.391x − 0.461).

A comparative determination of α-amylase activity at two temperatures with 24 sera ranging from 19.6 to 642 U/l (test B, 25 °C) yielded 30 °C/25 °C ratios from 1.26 ± 0.11 (test A1) and 1.26 ± 0.10 (test A2) to 1.34 ± 0.09 (test B) thus reflecting the preponderance of the pancreatic isozyme in those specimens (see tab. 5).

Table 8 presents a survey of all statistical data from parallel investigations of sera and urines with different methods. For regression analysis the data underwent log transformation because of their apparent log-*Gaussian* distribution in all assays. Their correlation was fairly good as shown graphically (fig. 1 and 2), and correlation coefficients calculated by nonparametric tests, like those from parametric tests (tab. 8) (*Wilcoxon*'s signed ranks test and *Spearman*'s rank correlation) were also above 0.910. However, we rarely detected urines which displayed extremely low α-amylase activites in the maltotetraose procedure but quite normal values with all techniques employing α-glucosidase. Hence, in these cases we presumed erroneous results with the maltotetraose procedure due to the presence of inhibitors which were dialysable but not yet identified.

Conclusions

To characterize in brief the essential qualities of α-amylase measurements with nitrophenyl glucosides, it can be stated that these substrates offer advantages

of shorter lag phases and a lack of dependence on NADH producing and consuming metabolites and/or enzymes of the sample, thus yielding a real, low and constant blank rate, which cannot always be guaranteed by other methods (30). Moreover the unfavourable preference of the salivary isozyme by the maltotetraose procedure is reduced even though not abolished by the use of nitrophenyl glucosides that are more sensitive to animal amylases.

As for linear reaction rates (concerning dilution range and duration of measurement) all assays compared are almost equal. α-Glucosidase seems to be more resistant to some inhibitors that very occasionally in urines than the three-step enzyme system of test B. However, the chromogenic substrates present problems in the measurement of highly active sera, mostly due to the influence of protein on the absorptivity of 4-nitrophenol. Further, their defined degradation products (34) might change with reaction medium and sample constituents. Although these methods have promoted a definite progress in the assay of α-amylase, two postulates from our expert group (44) are still far from realization, viz. the absence of lag phases and of deviations from linearity.

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Tab. 9. Intermethod comparison of α-amylase assays at 25 °C. The ciphers denote: range of values for different materials (U/l with test B), number of paired samples (n), linear regression equation with test B (maltotetraose method) as independent variable x (slope and y-intercept), and *Pearson* correlation coefficient (r).

Range of values U/l	n	Dependent variable y	Regression equation y = ax + b	r
Clear sera				
5.4–902	135	4-Nitrophenyl-α-*D*-maltoheptaoside (A 1)	y = 4.71x – 27.6	0.926
5.4–902	135	4-Nitrophenyl-α-*D*-maltopentaoside/hexaoside (A 2)	y = 1.80x – 4.81	0.932
5.4–902	135	Maltoheptaose (C 1)	y = 4.78x + 5.60	0.991
5.4–902	135	Oligosaccharides (C 2)	y = 3.92x – 12.1	0.993
Lipaemic sera (Triglycerides 4.10–51.4 mmol/l)				
15.9–197	10	4-Nitrophenyl-α-*D*-maltoheptaoside (A 1)	y = 5.48x – 57.0	0.983
15.9–197	10	4-Nitrophenyl-α-*D*-maltopentaoside/hexaoside (A 2)	y = 2.15x – 18.1	0.986
Icteric sera (Bilirubin 26.3–283 µmol/l)				
16.9–115	10	4-Nitrophenyl-α-*D*-maltoheptaoside (A 1)	y = 6.03x – 39.5	0.996
16.9–115	10	4-Nitrophenyl-α-*D*-maltopentaoside/hexaoside (A 2)	y = 2.02x – 6.21	0.999
Urines (Glucose 0–330 mmol/l)				
4.7–120	40	4-Nitrophenyl-α-*D*-maltoheptaoside (A 1)	y = 4.25x + 8.68	0.909
4.7–120	40	4-Nitrophenyl-α-*D*-maltopentaoside/hexaoside (A 2)	y = 1.84x + 2.18	0.979
4.7–120	40	Maltoheptaose (C 1)	y = 4.90x + 12.1	0.970
4.7–120	40	Oligosaccharides (C 2)	y = 3.36x + 18.8	0.980

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